Human Skeletal Growth Factor: Characterization of the Mitogenic Effect on Bone Cells in Vitro†

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ABSTRACT: The preceding paper [Farley, J. R., & Baylink, D. J. (1982) *Biochemistry* (preceding paper in this issue)] detailed the purification of a potent growth factor from human bone matrix. The purified protein (M_r 83 000) was shown to increase, in vitro, both the proliferation rate of embryonic chick calvarial cells and the growth rate of embryonic chick bone. We now report that the effect of this human skeletal growth factor on chick calvarial cell proliferation in vitro is saturable, prolonged, and inductive. The effect was saturable in that dose/response studies revealed a $K_{m_{app}}$ of 12.5 nM/10^3 cells in a 1-mL volume. The absolute value of $K_{m_{app}}$ varied with initial cell density. Although the peak response for DNA synthesis occurred after 16–22 h of exposure to the purified factor (508% of control, $p < 0.001$), DNA synthesis was still elevated (280% of control, $p < 0.001$) after 66 h of continuous exposure. The peak response seen after 16–22 h was evident without the continuous presence of the factor (i.e., when cells preincubated with the factor for 5–6 h were incubated in factor-free medium), suggesting that the effect is inductive. Actual counts of calvarial cells in culture revealed that in response to added factor, both cell number and maximum cell density (161% of controls, $p < 0.001$) were increased. This factor was also shown to stimulate chick calvarial cell proliferation in confluent cultures (185% of control, $p < 0.001$). The effect on calvarial cell proliferation in subconfluent cultures was not sensitive to indomethacin, but was subject to competitive inhibition by isobutylmethylxanthine and noncompetitive inhibition by trifluoperazine (a calmodulin antagonist). The factor also increased the proliferation rate of human bone cells in vitro, to as much as 1090% of serum-free controls. Preliminary studies suggest that the concentration of the factor in trabecular bone is greater than the concentration in cortical bone ($p < 0.001$).

In the preceding paper (Farley & Baylink, 1982) we described the purification of a skeletal growth factor from demineralized human bone matrix. The substance was characterized as a large molecular weight protein (M_r 83 000) with the ability to (a) increase the proliferation rate of chick calvarial cells in culture and (b) enhance the growth of cultured embryonic chick bone at concentrations much lower than those required for less specific supplements such as FCS[1] or BSA. Since previous studies from this laboratory had shown that the coupling of bone formation to resorption is subject to local control (Howard et al., 1980) and is mediated, at least partially, by an increase in osteoblast number (Baylink & Liu, 1980), both the presence of this factor in bone matrix (where is could be released by resorption) and its activities on bone cell proliferation and bone growth in vitro are consistent with its possible function as a coupling factor in vivo. In the current studies, we sought to characterize the activity of purified hSCF on bone cell proliferation in vitro, using both embryonic chick and adult human bone cells.

Experimental Procedures

Chemicals and Supplies. Tissue culture supplies were purchased from Gibco. [^{3}H]Thymidine was obtained from New England Nuclear; IBMX, indomethacin, tetrodotoxin, and BSA were purchased from Sigma. PTE was obtained from Lilly. Trifluoperazine was a gift from Smith, Kline & French.

Isolation and Culture of Embryonic Chick Calvarial Cells. This procedure has been described in detail (Drivdahl et al., 1980a). Briefly, a population of cells was prepared from 16-day-old embryonic chick calvaria by sequential collagenase digestion. The cells were plated at an initial density of 250–350/mm^2 in 16-mm diameter multiwell plates in a 1-mL volume of serum-free BGJ_b medium. Cells were cultured overnight before addition of effectors as described below.

Assay for Cell Proliferation. The proliferation rate of cells in monolayer culture was assayed by the incorporation of ^{3}H-TdR into Cl_3CCOOH-insoluble material during a 2-h incubation. The assay was adapted from existing methodology (Gospodarowicz et al., 1978) and has been detailed elsewhere (Puzas et al., 1981). Briefly, the chick calvarial cells were plated as described above, cultured overnight, and then exposed to effectors for an 18-h incubation, unless noted otherwise. Regardless of the incubation time, ^{3}H-TdR (1 μCi/mL) was added to the cells for the final 2 h of the culture period, and then the radioactive medium was removed, the cells were rinsed (cold phosphate-buffered saline), and a Q-tip, moistened in 12.5% Cl_3CCOOH, was used to swab each culture well. This process traps cellular material within the cotton fibers. The cotton tips were then washed twice in 12.5% Cl_3CCOOH and once in 95% ethanol (10 min for each wash) and counted in scintillation vials. A minimum of six wells were assayed for each sample (i.e., six separate data points for each determined mean). This assay assumes that de novo synthesis of DNA in our culture system reflects cell proliferation. In support of this assumption, hSGF increases cell number as well as ^{3}H-TdR incorporation (see Results).

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[1] Abbreviations: hSGF, human skeletal growth factor; cSCF, putative chick skeletal coupling factor; ^{3}H-TdR, tritiated thymidine; FCS, fetal calf serum; 25-OHD_3, 25-hydroxyvitamin D_3; 24,25-OH_2D_3, 24,25-dihydroxyvitamin D_3; 1,25-OH_2D_3, 1,25-dihydroxyvitamin D_3; IBMX, isobutylmethylxanthine; PTE, parathyroid extract; Cl_3CCOOH, trichloroacetic acid; BSA, bovine serum albumin; HSA, human serum albumin; EDTA, ethylenediaminetetraacetic acid.

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One unit of hSCF activity is defined as the amount of material required to obtain a 100% increase in the cell proliferation rate (above control values) when chick calvarial cells are plated at 350/mm^2 and ^{3}H-TdR incorporation is assayed 16–18 h after addition of the factor; 1% FCS was used as a positive control for cell responsiveness and was found to increase ^{3}H-TdR incorporation to 280–300% of control. Chick cells were prepared at weekly intervals for these studies, and the variation between assays was usually greater than the variation within assays. We have therefore only made comparisons between results obtained with the same preparation of cells. Within assay variation was usually about 10%; examples of variation within the controls are given in the figure legends.

Purification of hSGF. This procedure is described in detail in the preceding paper (Farley & Baylink, 1982). Briefly, human femoral heads (obtained at hip replacement surgery) were pulverized and rinsed thoroughly (to remove contaminating serum and marrow) before demineralization by dialysis in 10% EDTA at pH 7.0. The protein solution thus obtained from demineralized human bone matrix contained hSGF activity based on its ability to increase ^{3}H-TdR incorporation. This crude factor was purified to homogeneity by selective heat and acid precipitations, Sephadex G-200 column chromatography, and preparative polyacrylamide gel electrophoresis.

Protein concentration was measured by the dye-binding method of Bradford (1976), and hSGF activity was determined by reciprocal analysis of cell proliferation data (^{3}H-TdR incorporation); 1/% stimulation was plotted vs. reciprocal factor concentration, and activity was calculated from the apparent K_m (e.g., the concentration required to obtain half the maximal effect). The kinetic nomenclature of Segel (1975) is used throughout.

Isolation and Culture of Human Bone Cells. Cells were prepared from human bone samples (anterior iliac crest biopsy from a patient with secondary hyperparathyroidism, and a femoral head obtained at hip replacement surgery) by collagenase digestion (Worthington; type II, 2 mg/mL). The cells released during a 120-min incubation at 37 °C were rinsed and plated in 16-mm diameter multiwell culture dishes (Falcon) at an initial density of 30–200/mm^2 in BGJ$_b$ medium supplemented with 1% FCS. The cells were grown to confluence (14–24 days) and subcultured, using the same medium. Third to fifth passage cells were used for these studies, and the cells were rinsed and changed to serum-free BGJ$_b$ medium (or medium with supplements as described) 1–3 days before the addition of effectors. We believe that the cells in our cultures are bone cells and, more specifically, members of the osteoblast cell line because of the following observations: (1) The cells obtained from the biopsy cylinder were alkaline phosphatase positive, as assessed by enzyme cytochemistry (Gruber et al., 1982), even after several passages (the cells from the femoral head were not tested). This enzyme is regarded as a marker in bone for osteoblasts and their precursors (McComb et al., 1979). (2) Although leukocytes also stain for alkaline phosphatase activity and these cells are potential contaminants in our preparations, no leukocytes were observed in our cultures. Furthermore, recent studies have shown that when cells are prepared from rabbit bone marrow (including the outer endosteal layers), the hematopoietic cells do not survive the first 2 weeks of culture (Ashton et al., 1980). (3) The same studies report that the stromal cells that survive in culture begin to look fibroblastic and lose alkaline phosphatase activity. Even at this point, however, they will redifferentiate in diffusion chambers in vivo, becoming alkaline phosphatase positive and forming bone and cartilage (Ashton et al., 1980). Since fibroblastic cells derived from spleen will not form bone under the same conditions (Friedenstein et al., 1970), it seems clear that bone cells or bone cell progenitors can be cultured from mammalian bone. (4) Human bone cells (i.e., the cells obtained from bone biopsy samples) but not human skin fibroblasts were found to metabolize 25-OHD$_3$ to 1,25-OH$_2$D$_3$ in vitro (Howard et al., 1982). (The bone cells obtained from the femoral head were not tested.) Similarly, previous studies from this laboratory have established that chick calvarial bone cells but not chick skin, muscle, or liver cells possess this 1α-hydroxylase activity in vitro (Turner et al., 1980).

Partial Purification of hSGF from Trabecular and Cortical Bone. Each of four human femoral heads was dissected to provide multiple samples of normal trabecular and cortical bone free of articular cartilage (sample size was normalized by weight). The samples were rinsed with jets of water, frozen in liquid nitrogen, crushed, and repeatedly rinsed to remove contaminating serum and marrow before demineralization by dialysis in 10% EDTA at pH 7.0. (Samples for calcium determination were ashed but not demineralized.) After 21 days of dialysis, the extracted hSGF activity was partially purified by heat and acid precipitations as previously described (Farley & Baylink, 1982). Activity was determined from values of $K_{m_{app}}$ obtained by reciprocal analysis of cell proliferation data and expressed as units per gram wet weight of bone. Samples were also analyzed for calcium content, hydroxyproline content, and extractable protein concentration so that activity could be compared at equal protein concentration, and expressed as units (^{3}H-TdR incorporation) per unit of bone matrix (hydroxyproline) or bone mineral (Ca). Calcium was determined on duplicate samples by ionized absorption spectrometry (Willis, 1960), and hydroxyproline was determined (also in duplicate) by automated analysis of acid-hydrolyzed samples according to the method of Prockop & Udenfriend (1960).

No additional hSGF activity was released by a longer demineralization phase (7–14 additional days) nor was any additional activity released by digesting the matrix with collagenase. This could not be explained by inactivation of factor with collagenase, since we have already shown that hSGF is not affected by collagenase (Farley & Baylink, 1982). Although we cannot dismiss the possibility of protease contamination, and hSGF is sensitive to trypsin, we assume that protease activity in the demineralizing bone extract would be minimal in the presence of 10% EDTA. (The collagenase digestion of the matrix residue was not performed in 10% EDTA.) Furthermore, the samples of cortical and trabecular bone were processed identically. Thus, we have no reason to suspect differential recovery from cortical and trabecular bone. The validity of any difference between cortical and trabecular bone will be uncertain, however, until recovery is directly monitored.

Statistical Analysis of Data. All data are given as mean ± standard deviation except where otherwise noted, and comparisons were made by using Student's t test for significance.

Results

Characteristics of hSGF Activity on Chick Calvarial Cells. Preliminary studies, shown in Figure 1, were required to determine the effects of initial plating density and time in culture on the response of chick calvarial cells to purified hSGF. The data in Figure 1a demonstrate two interesting features of this system. (1) The number of cells that adhere to the plate and divide, reflected by the amount of ^{3}H-TdR incorporation, is

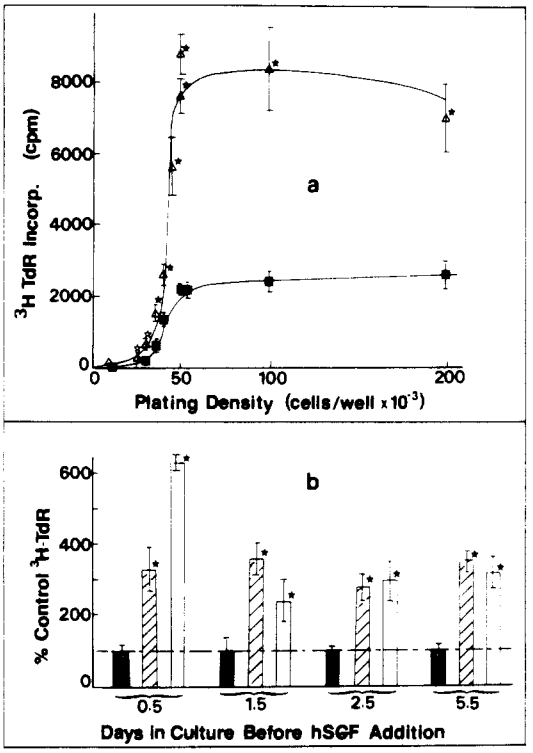

FIGURE 1: Effect of cell density on hSGF activity. (a) ^{3}H-TdR incorporation vs. initial plating density. Chick calvarial cells were plated in 16-mm diameter wells at the indicated concentrations, cultured overnight, and then grown with (△) or without (■) added hSGF (10 μg/mL) for an 18-h incubation. ^{3}H-TdR incorporation was assayed during the final 2 h of this culture period. All values are given as the mean ± SD of six separate determinations; (☆) a significant difference from minus hSGF controls, $p < 0.01$; (★) $p < 0.001$. (b) Percent of control ^{3}H-TdR incorporation vs. days in culture before hSGF addition. Calvarial cells were plated at 70 000/16-mm diameter well (350 cells/mm^2) and cultured for an 18-h period, beginning at the indicated time, in the presence of 10 μg/mL added hSGF (diagonally striped bars), 1% added FCS (open bars), or no additions (solid bars, controls). ^{3}H-TdR incorporation was assayed during the final 2 h of exposure. Values are presented as the mean ± SD of six separate determinations; (★) significant difference from controls, $p < 0.001$.

not a linear function of initial plating density. Subsequent studies, in which cells were plated at different densities, allowed to attach, then rinsed, and counted, revealed that plating densities of 35 000, 70 000, 140 000, 300 000, and 500 000 cells per 16-mm diameter multiwell resulted in plating efficiencies of 2.9%, 4.0%, 10.2%, 21.0% and 22.2%, respectively. (2) The data in Figure 1a also reveal that the effect of purified hSCF on calvarial cell proliferation after 18-h exposure is independent of initial plating density over the range 30 000–200 000 cells/well (i.e., the percent increase in ^{3}H-TdR incorporation was constant). In subsequent studies, cells were usually plated at 70 000/well. The data in Figure 1b demonstrate that the effect of hSGF on cells plated at 70 000/well (350 cells/mm^2) was independent of time in culture for up to 5.5 days.

The time course for the response of calvarial cells to hSGF was determined on cells plated at 350/mm^2 and cultured for 66 h with hSGF present continuously for the final 4–66 h. ^{3}H-TdR incorporation was measured during the final 2 h of culture. As shown in Figure 2, the peak of hSGF activity occurred between 14 and 22 h of exposure, corresponding to the peak of stimulation seen with 1% BSA. In these cultures, DNA synthesis was still significantly increased after 66 h of continuous exposure to hSGF, while the effect of 1% BSA did not extend beyond 24 h. (BSA did not show a significant effect at doses less than 100 μg/mL.)

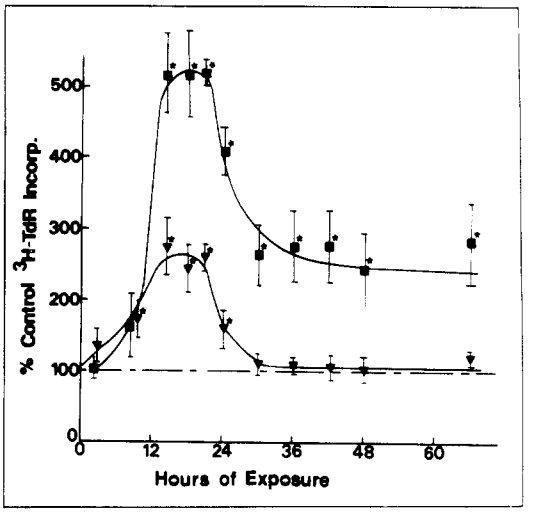

FIGURE 2: Time course of hSGF activity. Percent of control ^{3}H-TdR incorporation vs. hours of exposure. Calvarial cells were plated at 350/mm^2 and cultured in the presence of 10 μg/mL added hSGF (■) or 1% added BSA (▼) for 2–66 h. ^{3}H-TdR incorporation was assayed during the final 2 h of each incubation and compared to controls without additives. Values are shown as the mean ± SD of six separate determinations. The asterisk indicates a significant difference from control, $p < 0.001$, where control values ($n = 12$) were 100 ± 11%.

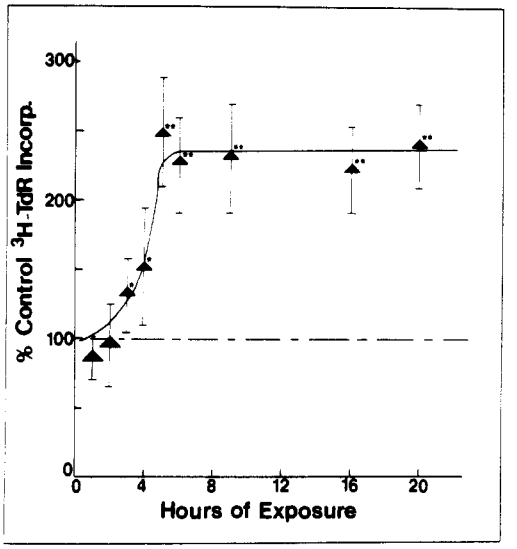

FIGURE 3: Effect of limited exposure to hSGF. Percent of control ^{3}H-TdR incorporation vs. hours of exposure. Calvarial cells were plated at 350/mm^2, cultured overnight, and then exposed to 10 μg/mL added hSGF for the initial 2–20 h of a 20-h culture period (controls had no added hSGF). ^{3}H-TdR incorporation was assayed during the final 2 h of culture. Values are shown as the mean ± SD of eight separate determinations. The asterisk indicates a significant difference from controls, $p < 0.01$; two asterisks indicate $p < 0.001$, where control values ($n = 16$) were 100 ± 13%.

In another experiment, calvarial cells (plated at 350/mm^2) cultured for 20 h and assayed for ^{3}H-TdR incorporation between 18 and 20 h were preincubated with hSGF during the first 1–20 h of incubation. These data are shown in Figure 3. While a 1- or 2-h exposure had no effect on cell proliferation at 18–20 h, a 6-h exposure was just as effective as 20 h of continuous exposure.

We next examined the effect of initial cell number (using the plating efficiencies given above) on the value of $K_{m_{app}}$ for hSGF enhancement of calvarial cell proliferation. These data are shown in Figure 4. Figure 4a shows a typical reciprocal analysis of 1/foldness of stimulation vs. reciprocal concen-

Table I: Potential Mediators of hSGF Activity

potential mediator	effector used	% control of ^{3}H-TdR incorporation[a]			
		effector alone	hSGF alone	effector + hSGF	significance + effector
cAMP	0.02 mM IBMX	80 ± 13%	461 ± 33%[b]	409 ± 45%[b]	NS
cAMP	0.10 mM IBMX	52 ± 6%[b]	275 ± 42%[b]	212 ± 31%[b]	$p < 0.01$
prostaglandin	50 μM indomethacin	101 ± 4%	275 ± 42%[b]	247 ± 12%[b]	NS
Ca^{2+}	1 μM trifluoperazine	63 ± 5%[b]	193 ± 23%[b]	181 ± 29%[b]	NS
Ca^{2+}	10 μM trifluoperazine	58 ± 11%[b]	193 ± 23%[b]	141 ± 20%[b]	$p < 0.01$
Na^+	1 μM tetrodotoxin	120 ± 21%	291 ± 49%[b]	234 ± 40%[b]	NS

[a] Standard cell proliferation assay (Experimental Procedures); values given as mean ± SD. [b] A difference from controls of $p < 0.001$. Values for hSGF alone are not constant because effectors were assayed at different times with different concentrations of hSGF.

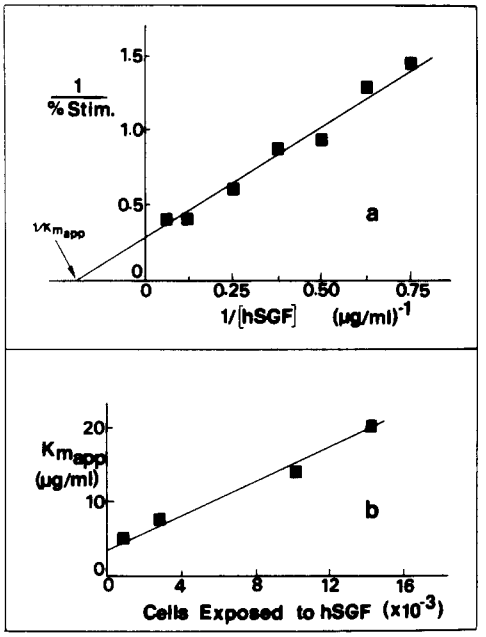

FIGURE 4: Kinetic analysis of hSGF activity. (a) Reciprocal foldness of stimulation vs. reciprocal concentration of hSGF. Calvarial cells were plated at 350/mm^2, cultured overnight, and then exposed to hSGF for 18 h. ^{3}H-TdR incorporation was assayed during the final 2 h of this period. Controls were assayed without added hSGF. The y axis intercept gives a value for the reciprocal of the (theoretical) maximum foldness of stimulation, and the x axis intercept affords a value for the reciprocal of $K_{m_{app}}$, the hSGF concentration needed to see half the maximum response. Values are shown as the mean of six separate determinations. (b) Replot of $K_{m_{app}}$ vs. initial cell density. $K_{m_{app}}$ was determined as above for hSGF activity on calvarial cells plated at different densities. Actual initial cell numbers were calculated by using the plating efficiencies given in the text.

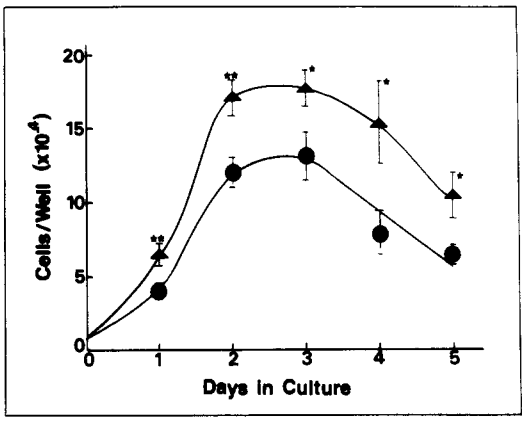

FIGURE 5: Effect of hSGF on cell proliferation. Calvarial cells were plated at 900/mm^2 and cultured for up to 5 days either with (▲) or without (●) added hSGF (10 μg/mL). At the indicated time, cells were quantitatively released from the plates and counted in a hemocytometer. Values shown are the mean ± SD of three pooled determinations. The asterisk indicates a significant difference from controls, $p < 0.05$; two asterisks indicate $p < 0.01$.

tration of hSGF, and Figure 4b shows a replot of values for $K_{m_{app}}$ vs. initial cell number. $K_{m_{app}}$ is dependent on cell number, and the slope in Figure 4b is about 1.05 μg/mL hSGF per 1000 cells, or 12.5 nM per 1000 cells.

Effect of hSGF on Confluent Cultures. In order to verify that the effect of hSGF was on cell proliferation as opposed to ^{3}H-TdR pool size, for example, cells were plated at 900/mm^2 (16-mm diameter wells) and cultured for up to 5 days. Cell number was determined daily by quantitatively recovering the cells from the plates (10–15-min exposure to 1 mg/mL collagenase and 1 mg/mL trypsin, 1:250 in 0.25% EDTA) and counting them in a hemocytometer. As shown in Figure 5, hSGF not only increased cell number but also increased the maximum cell density (161 ± 7% of control, $p < 0.001$). This maximum density was achieved after 2–3 days in culture, at which time the cells were confluent by microscopic examination. Thus, to test the effect of factor on maximum cell density, we prepared confluent cells by plating at 900/mm^2 and culturing for 3 days before the addition of hSGF. hSGF increased DNA synthesis in these confluent cultures of calvarial cells to 197% of control ($p < 0.001$). The same effect was also seen when calvarial cells were plated at 2300/mm^2 and cultured 18 h prior to the addition of hSGF (these cells were visibly confluent at this point): a 92% increase above control [^{3}H]thymidine incorporation values ($p < 0.001$).

Potential Mediators of hSGF Activity. The effects of potential mediators of hSGF-stimulated cell proliferation (i.e., cAMP, prostaglandin, Ca^{2+}, Na^+) were tested on calvarial cells, both alone and in combination with hSGF. The data are shown in Table I. IBMX, which should increase cellular cAMP, inhibited cell proliferation, at both 0.02 and 0.10 mM, but only at the higher concentration did it interfere with the effect of hSGF. Indomethacin (50 μM), which inhibits prostaglandin synthesis, and tetrodotoxin (1 μM), which inhibits Na^+ transport, had no effect. Trifluoperazine, a calmodulin antagonist (Cheung, 1980), inhibited cell proliferation and interfered with hSGF activity. Further analysis revealed that the effects of trifluoperazine and IBMX on hSGF activity were noncompetitive ($K_{i_{app}}$ = 6 μM) and competitive ($K_{i_{app}}$ = 70 μM), respectively. It is interesting to note that 1% FCS acted like a noncompetitive activator, with respect to hSGF activity (data not shown); 1% FCS increased $V_{m_{app}}$ but had no detectable effect on $K_{m_{app}}$.

Effects of hSGF on Human Cell Proliferation. As shown in Table II, hSGF increased the proliferation rate of cells from human bone in vitro. When crude hSGF was added to human bone cells prepared from the bone biopsy and to human skin fibroblasts (a generous gift of Dr. E. Bierman, Department of Medicine, University of Washington) in serum-free culture, it increased DNA synthesis in the bone cells to 1090% of

Table II: hSGF Activity on Cultured Human Cells

cell type[a]	culture conditions	concn of hSGF added (μg/mL)	effect[b] on ^{3}H-TdR incorporation	significance
bone cells, from biopsy	serum free	100 (crude)	1091 ± 203%[c]	$p < 0.001$
skin fibroblasts	serum free	100 (crude)	159 ± 67%	NS
bone cells, from femoral head	0.4% FCS	10 (pure)	212 ± 34%	$p < 0.002$
TE-85 osteosarcoma cells	serum free	20 (pure)	142 ± 20%[d]	$p < 0.002$

[a] All cells plated at about 200/mm^2. [b] Standard cell proliferation assay; values are percent of controls, mean ± SD; controls were grown serum free or with 0.4% added serum, as required. [c] Comparable to the effect of 0.6% added human serum; 5% human serum gave 3700 ± 552% of serum-free control. [d] Comparable to the effect of 1% FCS; these cells divide at 3–5 times the rate of normal human bone cells, even serum free.

Table III: Distribution of hSGF: Cortical vs. Trabecular Bone

parameter	cortical bone[a]	trabecular bone[a]	difference
hydroxyproline[b] (mg/g wet weight of bone)	26.25 ± 3.39	9.33 ± 2.66	$p < 0.001$
hSGF activity[c] (units/g wet weight of bone)	393 ± 113	405 ± 51	NS
relative yield of hSGF (units/mg of hydroxyproline)	14.97 ± 4.3	43.4 ± 5.47	$p < 0.001$

[a] Human femoral heads from hip replacement surgery were dissected to separate trabecular from cortical bone. Fragments were frozen, crushed, and rinsed as for normal preparation of hSGF (see Experimental Procedures). All values are shown as mean ± SD, $n = 4$. [b] 250–300 mg (wet weight) of unextracted bone was hydrolyzed in 12 M HCl for hydroxyproline determination of the autoanalyzer (Prockop & Udenfriend, 1960). [c] Activity in standard ^{3}H-TdR incorporation assay, determined by reciprocal analysis as described under Experimental Procedures.

controls, but had no effect on the skin cells. We later tested purified hSGF (at 10 μg/mL) on human bone cells obtained from a femoral head. As shown in Table II, even in the presence of 0.4% FCS (which was required for cell growth), hSGF caused a significant stimulation. The purified factor was also effective on serum-free cultures of osteosarcoma cells (TE-85, a generous gift from Dr. J. Fogh of the Sloan-Kettering Cancer Research Institute). The smaller magnitude of the response (e.g., 142% of controls) is probably due to the inherently rapid growth of the cells, even under serum-free conditions (e.g., 4–6 times normal ^{3}H-TdR incorporation on a per cell basis).

Source of hSGF: Trabecular vs. Cortical Bone. Trabecular and cortical bone samples, obtained from human femoral heads as described under Experimental Procedures, were analyzed for hSGF activity and for hydroxyproline content so the relative abundance of hSGF could be expressed in terms of bone matrix. The data, shown in Table III, suggest that hSGF is nearly 3 times more abundant in trabecular bone than in cortical bone, on the basis of hSGF activity per milligram of hydroxyproline. Nearly twice as much total protein was extracted from the trabecular bone as from the cortical bone (1.24 ± 0.09 mg/g wet weight vs. 0.63 ± 0.08 mg/g wet weight). The yield of calcium was 141 ± 19 vs. 253 ± 38 mg/g wet weight for trabecular and cortical bone, respectively.

Discussion

In the preceding paper (Farley & Baylink, 1982) we presented a model of coupling in bone which predicted (a) that skeletal coupling factor should be released in proportion to the extent of bone resorption, (b) that skeletal coupling factor should act as a mitogen for osteoblast progenitor cells, and (c) that skeletal coupling factor activity should result in increased bone formation. The growth factor we have purified from human bone matrix fulfills the latter two of these requirements. We have now shown that it stimulates the proliferation of chick calvarial cells, human bone cells, and human osteosarcoma cells in vitro, and we have previously shown (Farley & Baylink, 1982) that it stimulates the growth of embryonic chick bone. Further studies will be required to determine whether this factor actually mediates coupling in vivo; however, we have shown that the effect on cell proliferation is relatively specific for bone and cartilage cells.

We can now add the following observations to our understanding of this mitogenic effect: (a) The peak response for cell proliferation occurs after 16–18-h exposure to purified hSGF. This time course is comparable to what has previously been reported for epidermal growth factor (Canalis & Raisz, 1979) and cSCF (Drivdahl et al., 1980b). It is also similar to the time course we observed with milligram amounts of protein supplements like BSA. The fact that ^{3}H-TdR incorporation was still increased after 66 h of continuous exposure to the factor suggests that hSGF causes a prolonged stimulation of cell proliferation. Alternatively, we could argue that cell number was probably higher after 66 h of exposure to hSGF, compared to untreated controls, and this variable alone could result in increased ^{3}H-TdR incorporation per culture well. Against this alternative explanation is the observation that hSGF only increased cell number by 63% after 66 h, at which point ^{3}H-TdR incorporation was increased by nearly 200%, suggesting that the cell proliferation rate was still increased. The finding that cell number was increased much less than ^{3}H-TdR incorporation was not unexpected, since these two parameters are influenced by different processes. For example, cell number, but not ^{3}H-TdR incorporation, is directly influenced by cell death. (b) Purified hSGF stimulates cell proliferation in confluent cultures of chick calvarial cells and also increases maximum cell density. (c) The observations that $K_{m_{app}}$ increases with initial cell density and that the mitogenic effect is apparently inductive are consistent with the possibility that a second messenger, such as cAMP, prostaglandin, Ca^{2+}, or internalized receptors, mediates the effect of hSGF on cell proliferation. Accordingly, we tested the effects of indomethacin, IBMX, and trifluoperazine (as effectors of prostaglandin, cAMP, and calmodulin, respectively) in our assay system. Indomethacin did not inhibit the effect of hSGF on cell proliferation at 16–18 h, suggesting that prostaglandin is not essential for the effect. IBMX, however, did inhibit the response in a competitive manner, and trifluoperazine also inhibited, but in a noncompetitive fashion. These results suggest that a sufficient dose of hSGF can overcome the inhibitory effects of increased cAMP but cannot overcome a calmodulin blockade. While an increased concentration of cAMP clearly cannot be a second messenger for hSGF activity, the factor may act by inhibiting adenyl cyclase or otherwise decreasing the concentration of cAMP. Experiments are in progress to evaluate this possibility.

With regard to the distribution of hSGF in human bone, our data show that nearly 3 times as much activity is ex-

tractable from trabecular as opposed to cortical bone, when expressed as units per milligram of hydroxyproline. The difference is still 2-fold when expressed in terms of units per milligram of calcium, but the hydroxyproline content is probably a more appropriate index of bone matrix in these samples than is calcium. Although we need to directly monitor the recovery of hSGF in our demineralization/extraction method to verify these findings, the observation that more hSGF activity is extractable from trabecular than cortical bone could be significant in terms of regulation, particularly since we know that trabecular bone responds differently than cortical bone in human subjects with osteoporosis in response to fluoride therapy (Bang et al., 1978). We should note that these yields, about 400 units/g of bone, are in reasonable agreement with our previous estimate (Farley & Baylink, 1982) and correspond to a concentration of about 100 μg/g of bone. Because in adult humans the amount of bone resorbed per day is about 1.7 g (Harris & Heany, 1969; Wergedal & Baylink, 1974), we can estimate that about 170 μg of hSGF would be released per day. This would represent a very low local concentration at a specific resorption site, relative to the dose/response values given in Figure 4.

In summary, we have shown the effect of hSGF on bone cell proliferation in vitro is inductive and prolonged and may be mediated by some secondary mechanism. This mitogenic effect does not depend on the synthesis of prostaglandin or cAMP. The magnitude of the mitogenic effect depends on initial cell density as well as on hSGF concentration, and it can be observed in confluent cultures.

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Specific Activation of Particulate Leucyl-tRNA Synthetase Complexes†

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ABSTRACT: We have identified a specific activation of the high molecular weight form of leucyl-tRNA synthetase. The form at 30 S is activated 3–5-fold by a 2.5S cytoplasmic factor, which has no effect in the activity of the smaller 8S form of leucyl-tRNA synthetase. This 2.5S activator appears to have pyrophosphatase activity.

The key role of aminoacyl-tRNA synthetases in the translational process suggests these enzymes could be critical targets for regulatory factors. The existence of such factors has indeed been claimed by a number of laboratories [see Dignam & Deutscher (1979) for a review]. Dignam & Deutscher (1979) very nicely showed that most described aminoacyl-tRNA synthetase activators in the literature may be inorganic pyrophosphatase, whose mode of activation is simply to remove the very powerful aminoacyl-tRNA synthetase inhibitor inorganic pyrophosphate. The alanyl-tRNA synthetase activator described by Hilderman (1977), however, appears to be a real

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